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Our research program focus on novel observations to seek oncogenes in human breast cancer that will cooperate with stable expression of the angiogenic domains of pleiotrophin (PTN) and midkine (MK) to establish an "angiogenic switch" and, to identify downstream genes in the angiogenic pathway itself that are activated by the PTN and MK angiogenic domains. In the past year, we have developed MCF10A-PTN, -PTN mutant cell lines. Also, we have obtained an appropriate cDNA library from primary human breast cancers as a source of genes potentially able to use PTN to effectively enhance MCF10A cell growth in the nude mouse. The library is not derived from a cell line, but rather is derived from primary tissues, thereby avoiding the problems of mutations introduced in cell lines through prolonged passage. Furthermore, we have identified three downstream interactive proteins with the receptor protein RPTP $\beta/\zeta$  and thus of the PTN signaling pathway in human breast cancer cells.

We are pleased with progress thus far. We have established the base for obtaining results we sought in submitting the proposal. We have advanced understanding of pleiotrophin and midkine in the promotion of human breast cancer cells. We have identified and initiated understanding of the functions of pleiotrophin in these cells.

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## Introduction

Genetic mutations (or "switches") in malignant cells establish a more aggressive growth phenotype of the malignant cell and more aggressive tumor growth *in vivo* (Rastinejad, Polverini *et al.* 1989; Bouck 1990; Dameron, Volpert *et al.* 1994.). These switches are believed to activate previously quiescent pathways that have the ability to "cooperate" with (already) activated oncogenic pathways in the tumor cell to endow it with additional properties that favor tumor cell growth at the expense of the host. Genetic switches that signal an "angiogenic phenotype" are known as "angiogenic switches;" angiogenic switches are particularly devastating since neovascularization or "tumor angiogenesis" is believed to be rate-limiting for growth of malignant tumors *in vivo* (Folkman 1972; Maciag 1984; Folkman 1985) and for release of malignant cells to metastatic sites (Liotta, Kleinerman *et al.* 1974). This Idea Grant is directed to identifying and characterizing switches in breast cancer. It focuses on two related cytokines, pleiotrophin and midkine.

## Pleiotrophin

PTN is a PDGF-inducible, heparin binding growth/differentiation factor of 136 amino acids (Milner, Li *et al.* 1989). It is nearly 50% identical in amino acid sequence and shares identity of disulfide bonds with the protein product of *Mk* (Li, Milner *et al.* 1990), (Li, Gurrieri *et al.* 1992), a gene expressed in early stages of retinoic acid-induced differentiation in mouse embryonal carcinoma cells (Kadomatsu, Tomomura *et al.* 1988). *Ptn* and *Mk* are the only known members of gene family. Importantly, the *Ptn* gene is a proto-oncogene; fibroblasts transformed by *Ptn* develop highly vascularized tumors when implanted in the nude mouse (Chauhan, Li *et al.* 1993). PTN is highly expressed in many human tumors, including many human breast cancers; it is stably expressed in cell lines derived from these tumors (Fang, Hartmann *et al.* 1992; Wellstein, Fang *et al.* 1992; Tsutsui, Kadomatsu *et al.* 1993).

## Midkine

*Mk* also is a heparin binding growth/differentiation factor of 121 amino acids (Vigny, Raulais *et al.* 1989; Tomomura, Kadomatsu *et al.* 1990; Tsutsui, Uehara *et al.* 1991), (Fu, Maminta-Smith *et al.* 1994) with similar properties to PTN. MK transforms NIH 3T3 cells and highly expressed in many human cancers, including, breast cancer (Garver, Chan *et al.* 1993; Tsutsui, Kadomatsu *et al.* 1993; Kurtz, Schulte *et al.* 1995; Nakagawara, Milbrandt *et al.* 1995; O'Brien, Cranston *et al.* 1996; Nakanishi, Kadomatsu *et al.* 1997). Of potential major importance, a truncated form of the *Mk* mRNA encodes MK residues 1-3, 59-121, presumably the result of alternative splicing. The mutant gene product has been identified in cancer cells and in resected specimens of a number of different human cancers, including breast cancer.

## The Pleiotrophin Family in Tumor Promotion and the Angiogenic Switch

PTN and MK are the only two members of a family of developmentally regulated cytokines. Both the *Ptn* and *Mk* genes are protooncogenes. They signal remarkably similar functional responses as described below. Both PTN and MK are potent angiogenic factors. Expression of either PTN or MK is high in specimens from many breast cancers and stable expression of their genes is seen in cell lines that are derived from the breast cancers that have high level *Ptn* or *Mk* expression, suggesting transcriptional activation of *Ptn* or *Mk* is a feature of many human breast cancers. Constitutive expression of *Ptn* and *Mk* in non-transformed NIH 3T3 cells is transforming and in cells that are already transformed both PTN and MK significantly increase growth and establish an angiogenic phenotype when the cells that express these genes are implanted in the nude mouse. Remarkably, domains within PTN and MK (preliminary) alone, independently of the transforming domain, also establish an angiogenic phenotype in cells that are already transformed.

## Preliminary Data

Preliminary data and experiments in progress have shown expression of PTN and MK contributed to aggressive growth and the angiogenic phenotype of human breast cancers. Additional support for this idea has been obtained in human breast cancer cells that constitutively express the endogenous PTN gene. As example, introduction of a dominant negative PTN effector strikingly reduces the ability of these cells to grow rapidly and to establish an angiogenic phenotype.

Our preliminary data pointed to the novel concept that acquisition of stable expression of pleiotrophin (PTN the protein, *Ptn* the gene) and midkine (MK) establish genetic switches in tumor cells that signal an aggressive growth phenotype with significant tumor angiogenesis. We also found that domains within PTN and MK which are separate from and independent of the "transforming domain" signal the angiogenic phenotype when they are stably expressed in tumor cells, leading to the novel paradigm that structurally and functionally independent "angiogenic domains" in PTN and MK establish the phenotype of the cytokine in conjunction with the transforming domain of each MK or in cooperation with a second activated "oncogenic pathway" in the transformed cell. The domains that signal the cytokine phenotype are not transforming themselves; they appear to require cooperation with activated oncogenic pathway that can be but is not restricted to the oncogenic pathway signaled by PTN itself.

## Novel Concepts

The Idea Grant exploits these novel concepts to 1.) seek oncogenes in human breast cancer that will cooperate with stable expression of the angiogenic domains of PTN and MK to establish an angiogenic switch and to 2.) identify genes "downstream" in the pathway activated by the PTN and MK angiogenic domains.

## Expected Results

The Expected Results of this research are 1.) the identification of novel genes that are activated in human breast cancer which signal the transformation event itself in human breast epithelial cells through the ability of the identified PTN/MK angiogenic domains to amplify the transformed phenotype of the activated oncogene in mammary cancers, and 2.) the identification of genes that are activated (i.e., downstream) in the signaling pathway of an angiogenic switch in human breast cancer cells.

## Preliminary Data Leading to this Proposal

We used deletional mutagenesis to establish the domains in PTN required to transform NIH 3T3 cells. It was found using plaque formation, colony formation in soft agar, tumor formation in the nude mouse, and intratumor microvessel density to measure at tumor angiogenesis that PTN residues 41-136 and 1-64 are highly transforming, indicating that PTN residues 41-64 (the "transforming domain") are required to transform NIH 3T3 and that PTN 41-64 requires either the C-terminus or the N-terminus but not both to transform NIH 3T3 cells. PTN 69-136 alone fails to transform NIH 3T3 cells (Zhang, Zhong *et al.* 1999).

## PTN Contains an Independent Angiogenic Domain.

Tumors from NIH 3T3 cells transformed by PTN 41-136 have greater intratumor microvessel density than tumors derived from cells transformed by PTN 1-64, suggesting that PTN 69-136 contains an angiogenic

domain. To test this suggestion, PTN 69-136, and the naturally occurring alternate splice product MK 1-3, 59-121 corresponding to PTN 69-136 were tested in NIH 3T3, SW13 (human adrenal carcinoma), and MCF 7 (human breast carcinoma) cells. Constitutive expression of PTN 69-136 and MK 1-3 59-121 failed to transform NIH 3T3 cells but markedly accelerated tumor growth of SW13 cells and MCF7 cells and increased intratumor microvessel formation in the nude mouse.

### **Dominant-negative effector of PTN reverses aggressive growth and angiogenic phenotype of human breast cancer cells.**

To further establish relevance directly to human breast cancer, a gene to encode a dominant-negative PTN mutant was stably expressed in human breast cancer MDA-MB-231 cells that constitutively express high levels of the endogenous *Ptn* gene; the dominant negative PTN significantly reversed in the aggressive growth phenotype and the tumors in nude mice had reduced tumor angiogenesis, a result strongly supporting the role of PTN as a potent tumor promoter and angiogenic switch in some human breast cancers.

### **Hypotheses**

PTN and MK and their angiogenic domains alone (PTN 69-136, MK 1-3, 59-136) can cooperate with activated oncogenic pathways in human breast cancers to promote aggressive growth in human breast cancer and to act as angiogenic switches in these cells.

### **Progress**

#### **Objective I**

Progress has been made to meet Objective I.

In the original proposal, it was planned to prepare a cDNA library from MDA-MB-231 cells. This cell line was chosen because it was derived from a human breast cancer that expresses the endogenous PTN genes. Also, the dominant negative PTN mutant sharply reduces tumor aggression in these cells and their capacity to induce an angiogenic phenotype, establishing that PTN is accelerating growth of MDA-MB-231 cells.

We have now altered our strategy relative to the library to be used in these experiments. We now plan to use a cDNA library obtained from the National Cancer Institute. It is a cDNA library derived from four independent primary human breast cancers. We wish to take advantage of the fact that this library is an exceedingly well-made library by individuals interested in breast cancer and experienced in making cDNA libraries. It is a composite of four separate tumors, providing the potential for discovery of additional genes that will cooperate with pleiotrophin to convert the MCF 10A cells expressing the *Ptn* gene into a more aggressive growth phenotype. Furthermore, the library is not derived from a cell line, but rather is derived from primary tissues, thereby avoiding the problems of mutations introduced in cell lines through prolonged passage. This library has been obtained in the laboratory. We also have altered our strategy to attempt to place this library in a retrovirus system. The transfection efficiency of this library will be considerably higher using the retrovirus system and, we, potentially, bypass the need for selection.

We have transfected the angiogenic domains of PTN (69-136) and MK (1-3,59-121), and full-length PTN and MK, into the MCF10A cells, a human cell line derived from spontaneous immortalization of non-



malignant breast epithelium (Miller *et al.* 1993). Selection and characterization is ongoing. We anticipate moving ahead with completing clonal selection, expansion, and evaluation of the different levels of gene expression by Northern and Western analysis. Clones that show high-level expression will be retained, and cells will be tested for any changes in cell-cycle profiles, growth curves, and doubling times. Particularly important will be confirmation of previous preliminary results establishing that these cells do not form colonies in soft agar. This control is essential to ensure the effectiveness of the assay system for screening.

## Objective II

We have completed preliminary studies with the subtractive hybridization strategy originally outlined. We have not confirmed differential expression in the cells themselves. Nevertheless, in response to the critiques submitted to us at the time our project was awarded for funding, we have used alternative approaches to determine downstream effectors of PTN as well; this we believe has been most fruitful and is outlined below.

We used a modification of the recently described yeast two-hybrid system to identify genes whose protein products associate with the receptor protein tyrosine phosphatase  $\beta/\zeta$  (RPTP $\beta/\zeta$ ). RPTP $\beta/\zeta$  is the (a) receptor for pleiotrophin. It is expressed in MCF7 cells.

A yeast 2-hybrid design has several advantages for determining protein-protein interactions. First, it is an *in vivo* system that should maintain the native conformation of expressed proteins, thereby revealing authentic protein-protein interactions. Second, the genetic selection allows rapid screening of a large number of candidate proteins. Furthermore, this system facilitates the cloning and sequencing of cDNAs that encode interactive proteins with RPTP $\beta/\zeta$ . Finally, this lab has used this technique successfully to demonstrate other protein-protein interactions, providing expertise that will facilitate the analysis.

The full-length intracellular domain at RPTP $\beta/\zeta$  was used as "bait." After a yeast 2-hybrid screen using the Clontech Matchmaker System<sup>™</sup> (Clontech, USA), 292 colonies were seen under moderate selection (Trp<sup>-</sup>/His<sup>-</sup>). These 292 colonies were rescreened under the most stringent selective conditions (Trp<sup>-</sup>/His<sup>-</sup>/Ade<sup>-</sup>). Of these 292 colonies, 137 grew under the most stringent selective conditions. These 137 colonies were then assayed for  $\beta$ -galactosidase activity. Eighty-nine of the 137 colonies demonstrated  $\beta$ -galactosidase activity, as described above. The intensity of X-gal coloration was measured with a densitometer on the filter lifts and 31 of the colonies had moderate to intense blue staining (reflectance greater than 0.1). Twelve of the cDNA inserts have been sequenced and the remaining 19 clones are in the process of being purified and sequenced. After verification and characterization of these 31 clones, the clones with the lesser  $\beta$ -galactosidase activity were sequenced. No colonies were seen for the negative controls discussed previously.

From the 12 clones isolated encoding proteins interactive with the intracellular domain of RPTP  $\beta/\zeta$ , eight had greater than 95% nucleotide sequence identity to known human t-complex polypeptide (TCP-1). The other clones identified were: histone deacetylase 2 (HDAC2), transmembrane 4 superfamily member 81 (CD81), microtubule associated protein 1B (MAP1B), heat shock protein C041 (HSP041), and human adducin 2 $\beta$ .

We have chosen to focus initially on 3 of these clones, which encode TCP-1, HDAC2, and adducin 2 $\beta$ . The other clones encoding proteins of potential interest will be addressed subsequently as validation of their association with RPTP $\beta/\zeta$  by repeated sequence analysis and their criteria indicate their potential to participate in PTN signaling.

Since three of the independent, high affinity binding clones are t-complex polypeptide 1, the likelihood that this interaction is significant is high. The protein is a member of a hetero-oligomeric chaperone complex

localized to the cytosolic face of the Golgi apparatus. It is involved in maintaining the correct folding of cytosolic skeletal proteins, including actin and tubulin (Brown *et al.* 1996), and is involved in microtubule nucleation (Brown *et al.* 1996). Little is known about the regulation of TCP-1 mediated microtubule nucleation activity. It may also be an important step in the PTN signaling pathway and regulation of cell migration.

Another RPTP $\beta/\zeta$  binding protein uncovered is HDAC2. HDAC2 is potentially of major importance because of its role in silencing (Nagy *et al.* 1997). The HDAC family of enzymes deacetylates the histone 4 core protein, making the deacetylated region of nuclear chromatin inaccessible to transcriptional machinery. HDAC4 and 5 are sequestered within the cytoplasm by tyrosine phosphorylation at three sites (McKensey *et al.* 2000), allowing acetylated histones to remain in localized chromosome regions and "open" for gene activation. Since HDAC2 shares 60% amino acid sequence identity with HDAC4, it is possible that HDAC2 activity is also regulated by phosphorylation. If the RPTP $\beta/\zeta$ /HDAC2 interaction is shown to be valid *in vivo*, it is exciting to conjecture that RPTP $\beta/\zeta$  may be active in the regulation of the steady state levels of tyrosine phosphorylation of HDAC2, potentially regulating its translocation to the nucleus and the "shut down" of transcription of selective genes. Thus, it is speculated that sequestration of HDAC2 may be regulated through RPTP $\beta/\zeta$  phosphatase activity.

The other potential RPTP $\beta/\zeta$  binding partner selected is adducin 2 $\beta$ . Adducin 2 $\beta$  is localized at spectrin-actin junctions at the inner cell membrane and at sites of cell-cell contact in epithelial cells (Kaiser *et al.* 1989). Three reasons support its potential importance as a binding partner for RPTP $\beta/\zeta$ . It is localized to the inner cell membrane in cell types in which RPTP $\beta/\zeta$  is expressed. Adducin 2 $\beta$  also has a 13 amino acid tyrosine phosphorylation consensus motif and thus its phosphorylated form may be a substrate for RPTP $\beta/\zeta$ . Finally, adducin 2 $\beta$  is a cytoskeletal organizing protein and is a substrate for phorbol 12-myristate 13-acetate (PMA)-activated kinases, which function to activate adducin 2 $\beta$  ability to recruit spectrin and recruit actin filaments to the cell membrane (Kimwa *et al.* 1998). RPTP $\beta/\zeta$  may thus function in regulating these cytoskeletal elements in response to PTN signaling.

In support of the importance of these results, we have treated Hela cells with 50ng/mL PTN. Cells were probed with an antibody directed against phospho-adducin. The effect of PTN is clear; there is a striking loss of phospho-adducin associated with the nucleus and concomitant increase in cell membrane associated phospho-adducin. (See attached Figure).

These results are particularly exciting. Each of the proteins identified is expressed in MCF7 cells. Each of the proteins is involved either with cytoskeletal regulation and thus cytoskeletal stability, critical to metastasis or cell adhesion in breast cancer or, with gene transcription. The use of the different approaches thus is of major importance and seems likely to be very helpful in understanding roles of PTN in progression of human breast cancer.

We have initiated manuscripts to describe results of experiments in which PTN and different mutant PTNs have been introduced into MCF7 cells and studied as tumors in the nude mouse. These data have made clear both MK and PTN as well as the mutant proteins promote the growth of these tumors in the nude mouse.

We have also used "feeder cells" of NIH 3T3 cells co-mingled with MCF7 cells and showed the potential of these feeder cells to dramatically enhance the aggressiveness of the tumors at site of implantation of MCF7 cells. These studies are directed to understanding the roles of non-tumor cells and extracellular matrix in promoting the growth of breast cancer cells *in vivo*.



## **Key Research Accomplishments**

### **Objective I**

1. The achievement of an appropriate cDNA library from primary human breast cancers as a source of genes potentially able to use PTN to effectively enhance MCF 10A cell growth in the nude mouse.
2. The development of MCF 10A-PTN, -PTN mutant cell lines.

### **Objective II**

1. The identification of downstream interactive proteins with the receptor protein RPTP $\beta/\zeta$  and thus of the PTN signaling pathway in human breast cancer cells.

## **Reportable Outcome**

Manuscripts (in preparation) Pleiotrophin, Midkine and their Mutant Proteins Promote the Angiogenic Phenotype and Rapid Tumor Growth of Human MCF7 Breast Cancer Cells in Nude Mice.

## **Conclusions**

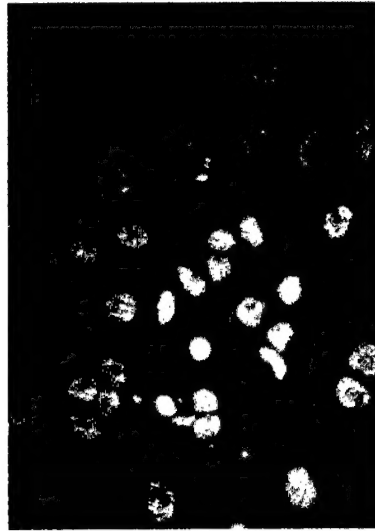
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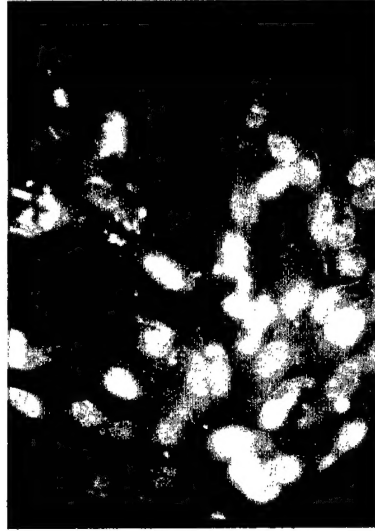
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**HeLa Cells Treated with 50ng of PTN and probed with phosphoadducin monoclonal antibody**  
**Photographs of light microscopy**



No PTN



15 minutes PTN treatment



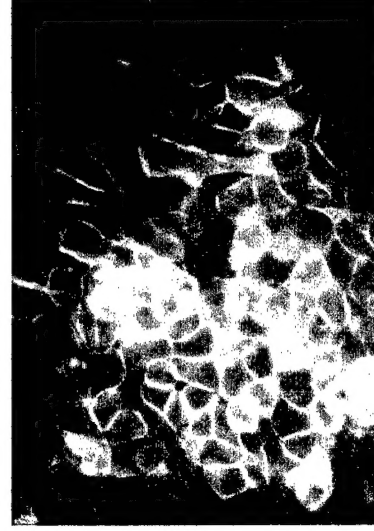
5 minutes PTN treatment



30 minutes PTN treatment



10 minutes PTN treatment



1hour PTN treatment